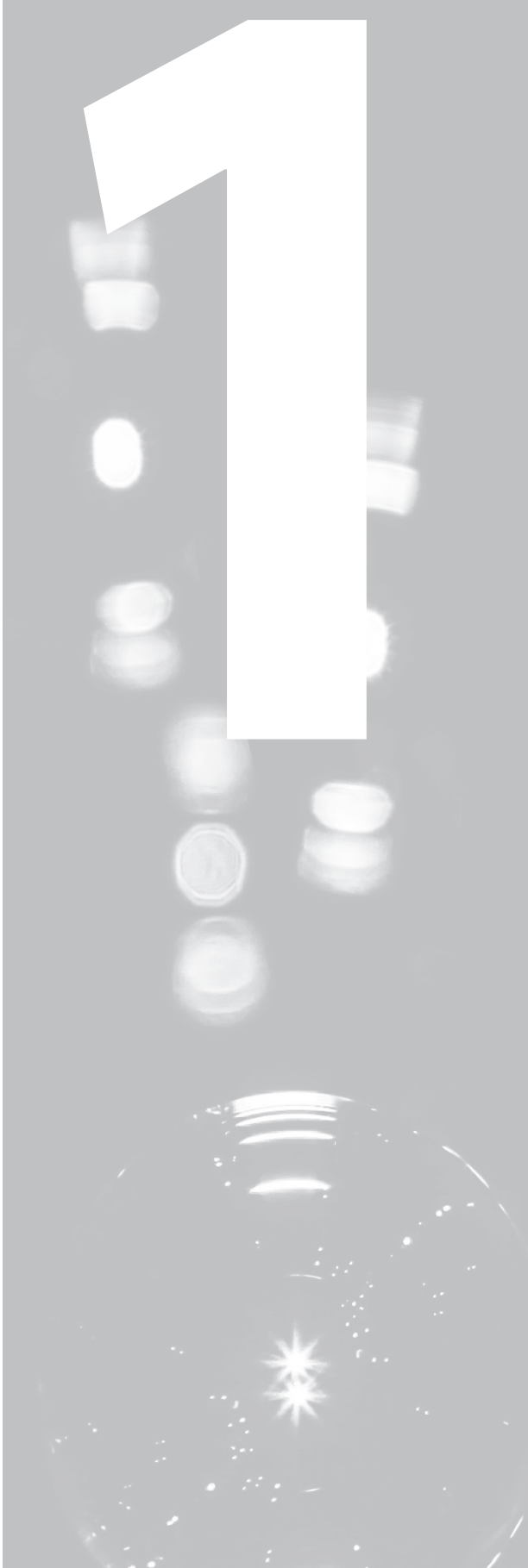
General introduction, scope and outline

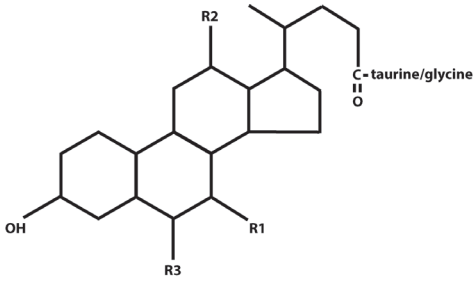


GENERAL INTRODUCTION

Bile salts

Bile formation and bile salt synthesis

The biliary pathway is one of the major routes through which endogenous waste products (*e.g*. bilirubin), drugs and other toxic compounds are eliminated from the body and also represents a main route for removal of excess cholesterol. Hence, formation of bile is an important physiological function of the liver. Primary bile is formed by hepatocytes and active secretion of bile salts (BS) into the bile canaliculi provides the main driving force for bile formation. Upon release in the small intestine, BS promote the absorption of dietary fats and fat-soluble vitamins. BS are synthesized from cholesterol in the hepatocytes by well-characterized biosynthetic pathways. The classical (neutral) pathway starts with 7α-hydroxylation of cholesterol by cholesterol 7α-hydroxylase (CYP7A1) while the alternative (acidic) pathway is initiated by sterol 27-hydroxylase (CYP27A1)[1]. Newly synthesized, so-called primary BS, are cholate (CA) and chenodeoxycholate (CDCA) in humans. In rodents, the relatively hydrophobic CDCA molecules are rapidly hydroxylated to form the more hydrophilic alpha- and beta-muricholates (α-MCA and β-MCA) (Fig. 1). Sterol 12α-hydroxylase (CYP8B1) activity is required for the biosynthesis of CA and determines the ratio CA to CDCA and, therefore, the hydrophobicity of the BS



	R1	R2	R3
CA	αOH	αOH	H
CDCA	αOH	H	H
DCA	H	αOH	H
LCA	H	H	H
UDCA	βOH	H	H
αMCA	αOH	H	H
βMCA	βOH	H	βOH
HCA	αOH	H	αOH
MDCA	H	H	βOH
ωMCA	βOH	H	αOH
HDCA	H	H	αOH

Figure 1. Bile salt structure
Structure of major naturally occurring bile salts. The primary bile salts in humans are CA; cholate and CDCA; chenodeoxycholate and the secondary bile salts in humans are DCA; deoxycholate, LCA; lithocholate and UDCA; ursodeoxycholate. The primary bile salts in rodents are CA, αMCA; alpha-muricholate and βMCA; beta-muricholate and the secondary bile salts in rodents are HCA; hyocholate, MDCA; murideoxycholate, ωMCA; omega-muricholate and HDCA;hyodeoxycholate.

pool [2]. The diversity of BS species in the BS pool is expanded by conjugation with amino acids to either taurine in rodents or glycine in humans in the liver and by the actions of intestinal bacteria that, *via* deconjugation, oxidation of hydroxyl groups and dehydroxylation, form the secondary BS deoxycholate (DCA) and lithocholate (LCA) in humans, and hyocholate (HCA), hyodeoxycholate (HDCA), murideoxycholate (MDCA), omega-muricholate (ω-MCA) and ursodeoxycholate (UDCA) in rodents (Fig. 1) [3]. These processes collectively creates a BS pool with specific physiochemical properties.

Enterohepatic circulation of bile salts

BS are maintained efficiently in the enterohepatic circulation (Fig. 2); 95% of the BS secreted by the liver is reabsorbed in the small intestine and only 5% is lost in the feces. The liver compensates for this loss by *de novo* BS synthesis. In the intestine, the reabsorption of bile salts is regulated through the expression of four important proteins, *i.e.*, apical sodium-dependent bile acid transporter (SLC10A2/IBAT/ASBT) [4], intestinal bile acid-binding protein (IBABP/FABP6) [5] and the heterodimer organic solute transporters alpha (SLC51A /OSTA) and beta (SLC51B /OSTB) [6], that are collectively responsible for the transport of BS from the intestinal lumen to the portal system. Na+-taurocholate cotransporting polypeptide (SLC10A1/NTCP) [7] and organic anion-transporting peptides (OATPs/SLCO superfamily) [8] are the major BS transporters at the basolateral membranes of hepatocytes for the uptake of BS and organic solutes from the portal system to the liver. Reabsorbed BS will be conjugated again and subsequently transported into bile by the bile salt export pump (BSEP/ABCB11) [9].

The Farnesoid X receptor

Nuclear receptors

Nuclear receptors are a class of proteins that are able to sense the cellular presence of nutrients, steroid hormones and certain other molecules like vitamins and BS. Upon activation, these receptors interact with other proteins to regulate the expression of target genes, thereby controlling a vast variety of cellular processes. Examples of nuclear receptors, their endogenous ligands and their main functions are the peroxisome proliferator-activated receptors (NR1C1/PPARα; NR1C2/PPARβ/δ;

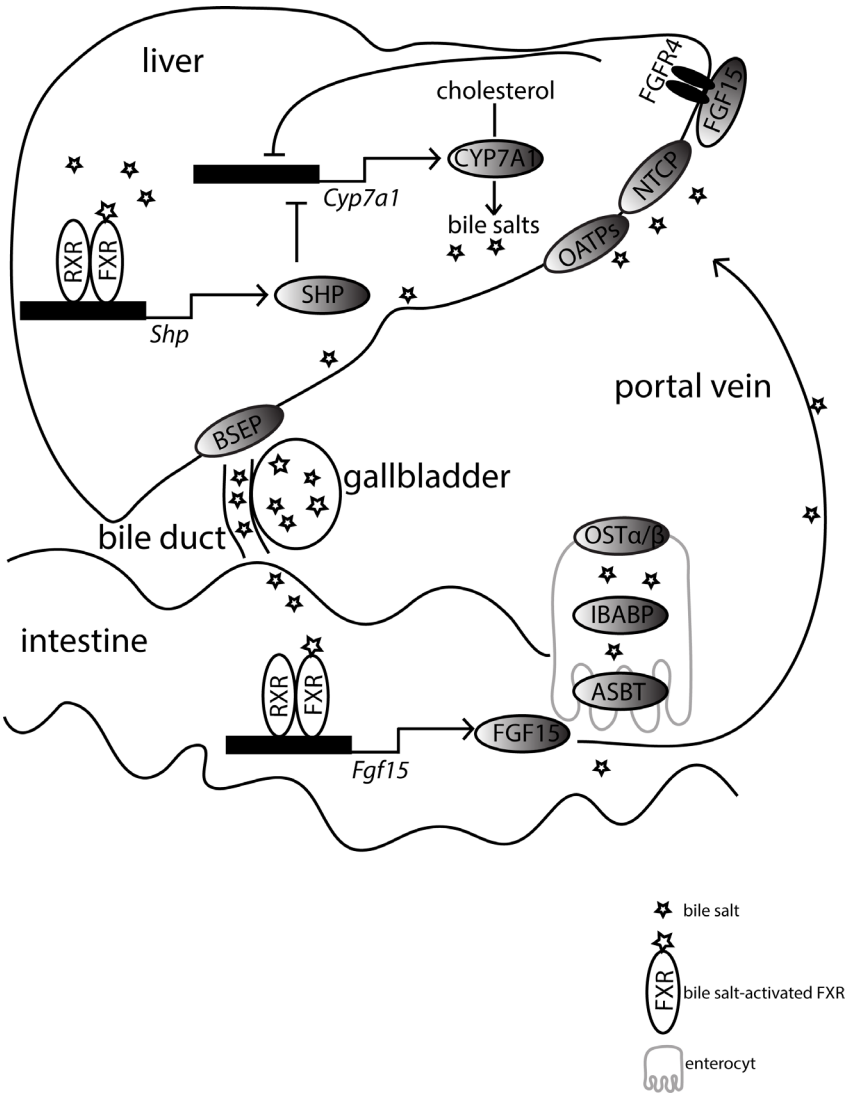


Figure 2. The enterohepatic circulation of bile salts
Bile salts (BS) are synthesized from cholesterol in hepatocytes and are actively secreted into the bile canaliculi that drain into the common bile duct. BS are stored in the gallbladder and upon ingestion of a meal the content is released into the intestinal lumen. In the ileum, BS are taken up via ASBT into the ileocytes and BS activate FXR which, together with RXR, induce the expression of Fgf15. FGF15 is released into the circulation and suppresses the hepatic BS synthesis by inhibiting Cyp7a1 expression. BS returning to the liver after reabsorption in the intestine also contribute to the negative feedback loop by suppression of Cyp7a1 via hepatic FXR activation. FXR; Farnesoid X Receptor, RXR; Retinoid X Receptor; CYP7A1; cholesterol 7α-hydroxylase, FGFR4; fibroblast growth factor receptor 4, FGF15; fibroblast growth factor 15, ASBT; apical sodium-dependent bile acid transporter, IBABP; intestinal bile acid-binding protein, OSTα/β; organic solute transporters alpha/beta, NTCP; Na+-taurocholate cotransporting polypeptide, OATPs; organic anion-transporting peptides.

NR1C3/PPARγ) that upon activation by free fatty acids function in control of cellular differentiation and lipid metabolism [10], Liver X Receptors (NR1H3/LXRα; NR1H2/LXRβ) that upon activation by oxysterols modulate cholesterol metabolism [11] and Farnesoid X Receptor (NR1H4/FXRα, from now on referred to as FXR [12-14] that upon activation by BS regulates steps in BS, lipid and glucose metabolism. The classical view of structural organization divided a nuclear receptor protein into 5 domains; the N-terminal transactivation independent regulatory domain (AF1), the DNA-binding domain (DBD), a flexible hinge region (H), a ligand-binding domain (LBD) and a C-terminal transactivation dependent domain (AF2) [15]. Nuclear receptors can bind DNA as homodimers, heterodimers or monomers [16]. Nuclear receptors have to ability to directly bind to DNA to regulate transcription or bind DNA as heterodimers with the common partner Retinoid X Receptor (NR2B1/RXRα; NR2B2/RXRβ) [17]. Upon binding to specific DNA sequences in the cell nucleus, nuclear receptors recruit transcriptional co-regulators, including transcription factors, co-activators, co-repressors and the RNA machinery itself, which all effect transcription [18].

Bile salt-activated receptor FXR

BS regulate their own synthesis in feed-back mechanisms involving FXR in the intestine and liver, the two organs in which FXR is highly expressed. FXR is the major BS responsive ligand-activated transcription factor and is involved in control of BS homeostasis. Intestinal BS-activated FXR induces the expression of fibroblast growth factor 19 (FGF19, and its mouse homolog Fgf15), which is secreted into portal blood to down-regulate hepatic BS synthesis *via* fibroblast growth factor receptor 4 (FGFR4) and B-Klotho signaling in the liver [19,20]. Activation of the FGFR4/B-Klotho (KLB) complex stimulates the c-Jun N-terminal kinase (JNK) pathway, suppressing hepatic *Cyp7a1* expression that catalyzes the rate-controlling step in the BS synthesis (Fig. 2). Hepatic BS levels are maintained by the control of uptake, metabolism, synthesis and excretion of BS. Activated hepatic FXR reduces *Cyp7a1* expression, in a short heterodimer partner (NR0B2/SHP)- and liver receptor homolog-1 (NR5A2/LRH1)-dependent manner [21,22]. Also *Cyp8b1* , determining the CA to CDCA ratio, and *Cyp27a1*, regulating the alternative BS biosynthetic pathway, are regulated by BS-activated FXR, in a SHP and hepatocyte nuclear factor-4 alpha (NR2A1/HNF4A)-dependent manner [23-26]. FXR can be activated by its endogenous ligands, *i.e.*, BS, with species-specific potencies. The relatively hydrophobic BS appear to be the most potent ones; CDCA>DCA>LCA>CA [13]. However, also several

synthetic compounds have been developed the past years, such as the BS derivative 6-ethyl-chenodeoxycholic acid (INT747) [27], but also non-steroid compounds such as GW4064 [28], WAY-362450 (XL335) [29], PX20350 and PX20606 [30].

FXR isoforms

FXR has four different splice variants in both humans and rodents [31,32]. These isoforms arise through alternative splicing of exon 5 and the use of distinct promoters that initiate transcription from either exon 1 or exon 3 (Fig. 3A and B). The different promoters of the *FXR* gene regulate the expression of FXRα1,2 or FXRα3,4 transcripts. FXRα1 and FXRα3 transcripts contain four amino acids (MYTG) in the hinge domain,

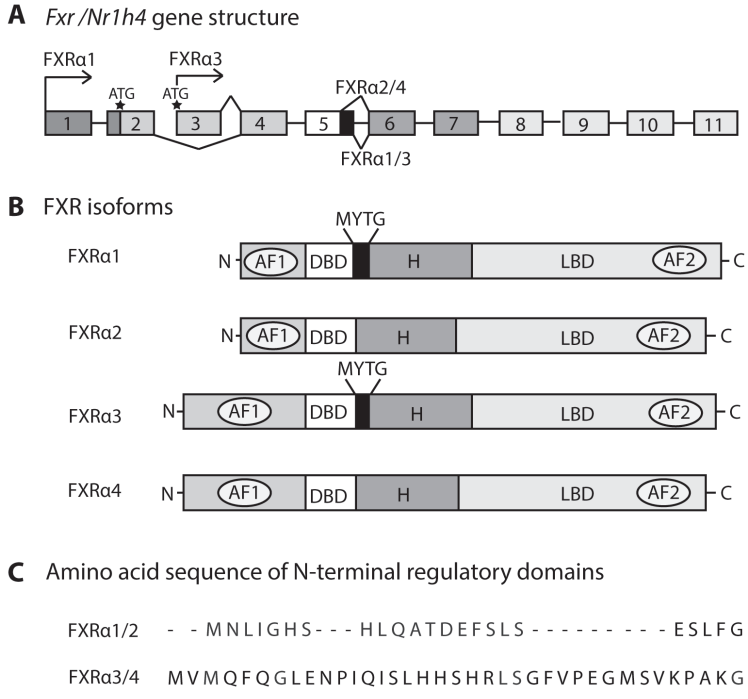


Figure 3. The Farnesoid X Receptor
Schematic representation of the murine Fxr/Nr1h4 gene (A) and FXR isoforms (B) with two distinct N-terminal regulatory domains (C). Two alternative promoters give rise to FXR isoforms FXRα1/2 and FXRα3/4 and a 12 base pair alternative splice donor site, which determines the difference between FXRα1/3 and FXRα2/4. AF1; transactivation independent domain, AF2; transactivation dependent domain, DBD; DNA binding domain; LBD; ligand binding domain, H; hinge region, MYTG; 4-amino acids insertion. –transcription start site, * translation start site.

adjacent to the DNA-binding domain (DBD), due to alternative splicing. The FXR isoforms show differences in spatial and temporal expression, as well as transcriptional activity [33,34]. In mice all four isoforms are abundantly expressed in liver, while FXRα3 and α4 are abundantly expressed in ileum, moderately in kidney and at low levels in stomach, duodenum, and jejunum. FXRα1 and α2 are moderately expressed in ileum and adrenal gland [32]. The difference between FXRα2 and FXRα4 lies within the N-terminal part of the protein, *i.e.*, resulting in a 37 amino acid extension of FXRα4 (Fig. 3C). This difference can cause conformational changes in the FXR protein itself and, thereby, influence the transcriptional activity and/or transactivation/repression capacity on target genes. A dozen transcriptional cofactors have been described to influence FXR transactivation [35], but actions specific for the FXR isoforms have not been described. The intriguing question remains why FXR isoforms exist and what their specific physiological roles in different tissues actually are.

The expression of FXR itself is tightly regulated, there are several cofactors that play a role in FXR-mediated transcription. PGC-1α , a well-characterized coactivator for PPARα and the master integrator of cellualr energy metabolism, coactivates FXR-target gene promoter activity *in vitro* [36-38]. Coactivators such as SMARCA4, SIRT1, EP300, CARM1, PRMT1 and ASCOM are recruited to FXR target genes, which results in histone modification or chromatin remodeling and increased gene transcription [39]. Interestingly, some of these cofactors, including EP300 and SIRT1, acetylate and deacetylate not only histones at FXR target genes but also FXR itself [39]. Molecular regulation of FXR activity also involves post-transcriptional phosphorylation, sumoylation and ubiquitination [39]. In addition, FXR expression levels vary with the nutritional status and appear to be tightly regulated by the metabolic status, indirectly supporting a role for this nuclear receptor in metabolic diseases [37,40-42] .

The relative abundance of hepatic FXRα3/4 was increased in children suffering from progressive familial cholestasis type I [43]. Also in cholangiocarcinomas [44] and colorectal adenocarcinoma [45] the relative abundance of hepatic and intestinal FXRα3/4 was shown to be increased. Apparently, disease states can be associated with a switch in FXR isoform expression in the liver and intestine which may influence outcome of the disease state progress. Recently, Vaquero *et al*. [46] reported that activation of human FXR depends on the pattern of FXR isoform expression and the BS composition. They showed that cell-specific pattern of FXR isoforms determine the overall tissue sensitivity to FXR agonists and may be involved in the differential response of FXR target genes to FXR activation.

Bile salt-activated FXR as metabolic regulator

BS-activated FXR turned out to be involved in control of lipoprotein, glucose and energy metabolism [47]. A number of these findings became evident from studies in FXR-deficient mice. FXR-deficiency was associated with reduced expression of the gluconeogenic enzyme PEPCK [48,49], impaired glucose tolerance, insulin resistance [50,51], hypertriglyceridemia and increased VLDL production [52,53]. Furthermore, administration of the natural FXR ligands, BS, resulted in increased energy expenditure in brown adipose tissue of mice fed a high fat diet [54]. Clinical observations showed that the treatment of patients with BS sequestrants, *i.e.* removal of natural ligands, also results in increased plasma triglyceride (TG) levels [55,56], while CDCA-treatment had the opposite effect [55,57,58]. The generation of synthetic FXR compounds provided new insights into the metabolic actions of FXR. *In vivo* treatment with the synthetic FXR agonist GW4064 significantly improved hypercholesterolemia, hypertriglyceridemia and hyperglycemia in mouse models of insulin resistance [48,50,51]. Furthermore, the synthetic FXR agonist PX20350/20606 induced HDL-mediated transhepatic cholesterol efflux in mice and monkeys and prevented atherosclerosis in a susceptible mouse model [30,59]. Note that BS are also able to activate the transmembrane receptor TGR5, also known as G-protein coupled BS receptor 1 (GPBAR1) and membrane-type BS receptor [60]. BS-activated TGR5 is involved in glucose metabolism, energy expenditure, immune response and liver function [61-63].

SCOPE AND OUTLINE

FXR is considered a promising target for novel therapies aimed at treatment of metabolic derangements associated with obesity, *i.e.* type II diabetes, non-alcoholic fatty liver disease and atherosclerosis. Activated FXR plays key roles in regulating metabolism of BS, lipids and glucose. The fact that four different FXR isoforms exist, and that these are conserved in rodent and human genomes argues for a functional role of these isoforms. The FXR isoform specific function regarding metabolic regulation is currently unknown and requires clarification.

Altogether, insight into tissue-specific actions of the FXR isoforms as well as in the consequences of alterations in BS pool size and composition are required to define optimal strategies to treat and prevent components of the metabolic syndrome. Using innovative mouse models and by modulating the activity of FXR,

we have evaluated their role in control of BS and cholesterol metabolism. **Chapter 2** reviews the metabolic effects of BS in the gut in health and disease. In **chapter 3**, we investigated the tissue-specific roles of FXRα2 and FXRα4, the transcriptionally most active splice variants of FXR, on BS biosynthesis and lipoprotein metabolism in the liver. We show distinct roles for FXRα2 and FXRα4 in the different branches of the BS biosynthetic pathway. Furthermore, mice with hepatic FXRα2 expression showed a relatively hydrophilic BS pool and increased their cholesterol turnover compared to FXRα4 mice. **Chapter 4** focusses on the pharmacological activation of both LXR and FXR, to overcome the hepatic triglyceride (TG) accumulation caused by pharmacological activation of LXR alone. We show that concomitant activation of both nuclear receptors ameliorated hepatic steatosis, possibly *via* increased TG-hydrolysis induced by pharmacological FXR activation. We also assessed the contribution of intestinal FXR on BS and cholesterol homeostasis, which is described in **chapter 5**. Our data revealed that intestinal FXR is capable of mediating removal of large quantities of cholesterol from the body. Stimulation of the TICE pathway was shown to contribute most to the increased cholesterol disposal in response to FXR activation. Furthermore, our data pinpoint intestinal FXR as a transcription factor impacting the hydrophobicity of the BS pool thereby governing the rate of transintestinal cholesterol excretion.

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